Research article

PnSAG1, an E3 ubiquitin ligase of the Antarctic moss *Pohlia nutans*, enhanced sensitivity to salt stress and ABA

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Plant U-box (PUB) E3 ubiquitin ligases play crucial roles in the plant response to abiotic stress and the phyto-hormone abscisic acid (ABA) signaling, but little is known about them in bryophytes. Here, a representative U-box armadillo repeat (PUB-ARM) ubiquitin E3 ligase from Antarctic moss *Pohlia nutans* (PnSAG1), was explored for its role in abiotic stress response in *Arabidopsis thaliana* and *Physcomitrella patens*. The expression of PnSAG1 was rapidly induced by exogenous abscisic acid (ABA), salt, cold and drought stresses. PnSAG1 was localized to the cytoplasm and showed E3 ubiquitin ligase activity in vitro ubiquitination assay. The *PnSAG1*-overexpressing *Arabidopsis* enhanced the sensitivity with respect to ABA and salt stress during seed germination and early root growth. Similarly, heterogeneous overexpression of PnSAG1 in *P. patens* was more sensitive to the salinity and ABA in their gametophyte growth. The analysis by RT-qPCR revealed that the expression of salt stress/ABA-related genes were downregulated in PnSAG1-overexpressing plants after salt treatment. Taken together, our results indicated that PnSAG1 plays a negative role in plant response to ABA and salt stress.

1. Introduction

Higher plants are frequently subjected to adverse environmental conditions, owing to either biotic factors (e.g., pathogens and herbivores) or abiotic factors (e.g., extreme temperature, water availability and high salinity), which severely affect plant growth and development. To survive under such (ever-changing environment) severe environmental stresses, sessile plants have evolved complicated protective strategies that involve integrated molecular, cellular, and metabolic programs (Hirayama and Shinozaki, 2010; Hummel et al., 2010). Stress signals are transduced to activate stress response genes and alter metabolic rates and ion channel permeability (Hasegawa et al., 2000). Among these strategies, the ubiquitin (Ub)-26S proteasome system, which mediates post-translational modification of proteins or degradation of the target protein, is one of the most prominent mechanisms during plants response to developmental cues or adaption to various environmental stresses (Smalle and Vierstra, 2004; Santner and Estelle, 2010; Stone, 2019).

E3 ubiquitin ligase, which delivers ubiquitin from the E2 (ubiquitin-conjugating enzyme) ubiquitin intermediate to the target protein, is the key factor in the ubiquitination system that determines the specificity of the extensive range of substrate (Vierstra, 2009; Sadanandom et al., 2012). Plants have larger numbers of different E3 ligases than other eukaryotes, with over 1400 different E3s in *Arabidopsis thaliana* genome and over 1300 members of E3 ligases in the rice genome (Stone et al., 2005; Du et al., 2009; Vierstra, 2009). According to the mechanism of action and the presence of special domains, E3 ligases can be largely categorized into four families: the E6-AP Carboxyl Terminus (HECT), Really Interesting New Gene (RING), Cullin (Cul)-RING ligases (CRLs) and U-box ligases (Smalle and Vierstra, 2004; Stone and Callis, 2007; Yee and Goring, 2009). Among them, the U-box domain contains 70 conserved amino acids and forms a tertiary scaffold structure resembling that of the (a modified) RING-finger domain (Azevedo et al., 2001; Ohi et al., 2003). Compared with the 2 U-box genes in *Saccharomyces cerevisiae* and 21 in human, there are 64, 77 and 125 U-box genes have been annotated in *Arabidopsis*, the rice and soybean genome, respectively, indicating that their function in diverse biological processes (Wiborg et al., 2008; Zeng et al., 2008; Wang et al., 2016).

U-box E3s from monocots and dicotyledons are involved in the growth and developmental processes of plants (Kinoshita et al., 2015; Somssich et al., 2016; Liu et al., 2018), plant innate immunity against pathogens (Ishikawa et al., 2014; Liu et al., 2015), and hormone regulation (Antignani et al., 2015; Jung et al., 2015; Zhou et al., 2018). In addition, a growing number of U-box E3s are also found to play roles in
regulating plants responses to abiotic stress. For example, the Arabidopsis PUB22 and PUB23 negatively regulate drought tolerance by facilitating pyrabactin resistance-like 9 (PYL9) degradation only when they interact with ABA receptors (Zhao et al., 2017). Rice U-box E3 ligases OsPUB2- and OsPUB3-overexpressing plants markedly enhanced cold tolerance in terms of survival rates, chlorophyll content, ion leakage, and the expression levels of cold-stress-inducible marker genes, suggesting that these genes may be highly valuable for improving crops resistance (Byun et al., 2017). The Arabidopsis PUB30 is a negative factor of salt stress response in seed germination through specifically ubiquitinating salt-tolerant gene BRI1 kinase inhibitor 1 (BKI1) and brassinosteroids signaling (Hwang et al., 2015; Zhang et al., 2017c). Overexpression of Glycine max GmPUB8 displays hypersensitivity to osmotic and salt stresses during seed germination and seedling growth, and inhibits ABA- and mannitol-mediated stomatal closure with less induction of drought stress-related genes (Wang et al., 2016). Triticum aestivum TaPUB1 improve germination and seeding survival rates, photosynthetic rate, water retention, as well as reduce the accumulation of reactive oxygen species (ROS) under drought stress, suggesting this gene positively modulates plant drought stress resistance potential by improving their antioxidant capacity (Zhang et al., 2017a).

Mosses are the most visually recognized organisms on extensive ice-free areas of Antarctica, and they are capable of growing and thriving under the physiologically extreme conditions on Earth (Pisa et al., 2013). Thus, the Antarctic mosses must have physiological and genetic characteristics that make them highly adapt to the harsh environment (Royles and Griffiths, 2015). For example, UV-B-absorbing compounds (UVAC) including red pigments within the cell walls have high photoprotection for Antarctic mosses surviving in multiple stresses (desiccation, naturally high UV and visible light) (Waterman et al., 2018). Overexpression of PaFKBP12, a peptidyl prolyl isomerase gene from the polar moss Polytrichastrum alpinum, resulted in more tolerance to heat, ABA, salt and drought stresses during root elongation and shoot growth in Arabidopsis (Alavilli et al., 2018). Several receptor like kinases, jasmonate ZIM-domain protein and genes of flavonoid synthesis pathways are involved in the adaptation of Antarctic moss Pohlia nutans to the polar environment (Wang et al., 2017a; Liu et al., 2019; Yao et al., 2019). In this study, we isolate a PUB-ARM ubiquitin E3 ligase gene from the Antarctic moss Pohlia nutans, which overexpression rendered bryophyte Physcomitrella patens and Arabidopsis plants hypersensitive to ABA and salt stress (PnSAG1). These results provide new insights into the functions of PUB proteins in Antarctica moss.

2. Materials and methods

2.1. Plant materials and stress treatments

Pohlia nutans was obtained from the vicinity of the Great Wall Station in Antarctica in March 2014. P. nutans was cultured in the pots soil containing Base Substrate (Klasmann-Deilman, Geeste, Germany) and local soil (1:1) and incubated at 16°C with 70% relative humidity and 70 μmol m\(^{-2}\) s\(^{-1}\) light. The mosses were treated with abiotic stresses including salinity (200 mM), dehydration (20% (w/v) polyethylene glycol 6000 solution), cold (4°C) and abscisic acid (ABA, 50 μM) for the indicated times. The aerial portions of the shoots were harvested and frozen immediately in liquid nitrogen after different stresses.

Arabidopsis thaliana used for wild type (WT) and transformation were grown on sterile soil and kept in the greenhouse at 22°C with 60% relative humidity and light intensity of 80 μmol m\(^{-2}\) s\(^{-1}\). After growing 4 weeks with 8 h light, they were transferred to 16 h light greenhouse for the plants transformation and maturation.

Physcomitrella patens were grown on BCD medium at 25°C with 60 μmol m\(^{-2}\) s\(^{-1}\) light. After 3 weeks with 16 h light, gametophyte tissues were sufficiently ground by a tissue homogenizer and then evenly distributed on cellophane overlaid BCD medium supplemental with 5 mM diammonium (+) tartrate to form protonema. The new protonema was used for the plants transformation.

2.2. Gene cloning and bioinformatics analysis

The HMMER program was used to search the ubiquitin ligases from the Antarctic moss P. nutans transcriptome (Finn et al., 2015). A full-length of gene ubiquitin ligases was selected for further analysis.

The protein sequence was analyzed using the SMART online software (http://smart.embl-heidelberg.de/) (Letunic et al., 2015). BLASTP web site was used to align multiple ubiquitin ligases sequences (Herrera-Galeano et al., 2014). The phylogenetic trees of ubiquitin ligases from P. nutans and other species were constructed using the Mega 6.0 program (Stecher et al., 2014).

2.3. RNA preparation and real-time polymerase chain reaction analysis

Total RNA was extracted from moss tissues or Arabidopsis seedlings using CTAB method and TRIzol reagent (Takara, Dalian, China) (Wang et al., 2017a). Single-strand cDNA was synthesized from 4 μg of total RNA with 5 × All-In-One RT MasterMix (abm, Canada). RT-qPCR was performed using the Bestar SybrGreen qPCR Master Mix (DBI Bioscience, Shanghai, China). The amplification procedures were initiated with a pre-denaturing step at 94°C for 2 min and followed by denaturing (94°C for 20 s), annealing (57°C for 30 s) and extension (72°C for 20 s) steps for 40 cycles during the second stage, and a final stage of 65–95°C to determine melting curves of the amplified products. The reference sequence was Ptnubulin, Ptnubulin or AtACTIN2. Data were analyzed using the 2\(^{-\Delta\DeltaCT}\) method (Livak and Schmittgen, 2001). All reactions were completed in triplicate. The relevant primer sequences for RT-qPCR assay are all presented in Table S1.

2.4. Subcellular localization of PnSAG1 proteins

The PnSAG1 coding sequence was PCR amplified using the primer pair (Table S1) and inserted into a pBl221:p35S:GFP vector to generate the transgene p3SS:PsAG1-GFP. Then the plasmid was purified and introduced into 4 week-old wild-type Arabidopsis mesophyll protoplasts as described by Yoo et al. (2007), and incubated overnight in the dark at 22°C. Meanwhile, the protoplasts were transformed with the pBl221:p3SS:GFP control vector. GFP signal and chlorophyll autofluorescence were detected using a confocal laser scanning microscopy at excitation wavelengths of 488 nm and 647 nm, respectively.

2.5. In vitro PnSAG1 activity assay

The PnSAG1, E1 (AtUBA1) and E2 (AtUBC8) coding regions were cloned into the PET28a vector with two His tag in C-terminal and N-terminal. The fusion proteins were expressed in Escherichia coli BL21 (DE3) strain and purified by affinity chromatography using Ni Sepharose High Performance (GE Healthcare) following the manufacturers’ instructions. Ubiquitination assays were carried out as previously described with some modification (Mark et al., 2016). Briefly, the reaction of 30 μl final volume contained 100 mM Tris-HCl, pH7.5, 25 mM MgCl\(_2\), 2.5 mM dithiothreitol (DTT), 20 mM ATP, 1 μg ubiquitin (sigma), 1 μg E1 (AtUBA1-His), 1 μg E2 (AtUBC8-His) and 1 μg E3 (PnSAG1-His). The reaction mixture was incubated at 28°C for 3 h and stopped by the addition of SDS sample buffer with boiling for 5 min. The sample was separated by 8% SDS-PAGE and subjected to Western blotting using the anti-ubiquitin antibody (New England BioLabs), and visualized by using an enhanced chemiluminescence detection system.

2.6. Generation of PnSAG1-overexpressing transgenic Physcomitrella patens and Arabidopsis plants

To generate the PnSAG1-overexpressing transgenic P. patens plants,
Fig. 1. Characterization and sequence analysis of PnSAG1.

a-b Amino acid sequence alignment of PnSAG1 with other U-box E3 ubiquitin ligases. c Phylogenetic relationship between PnSAG1 and other plant U-box proteins.
the PnSAG1 sequence was inserted into the pTFH15.3 vector according to the homologous recombination method as described by Zhang lab (Wang et al., 2017a). Before transformation, the pTFH15.3:PnSAG1 plasmid DNA was linearised by digestion with restriction enzyme Not I and purified by standard phenol extraction/ethanol precipitation, and dissolved at a final concentration of 2 μg μL⁻¹ sterile aqueous solution. 5d-old P. patens protoplasts were isolated following the modified protocol of Cove et al. (2009). Then the plasmid was purified and introduced into P. patens protoplasts using polyethylene glycol (PEG)-mediated DNA transformation method with some key modification (Hohe et al., 2004). Briefly, 30 μg of linearised pTFH15.3:PnSAG1 DNA fragments were mixed gently with 300 μL protoplast solution and 350 μL PEG solution (40% PEG 4000 and 0.3 M Ca(NO₃)₂ in 3M medium, pH5.6). The mixture was incubated at room temperature for 30 min and carefully diluted with the gradient volume of 3M medium every 5 min. After centrifuged for 10 min, the transformed protoplasts was resuspended in 3 ml regeneration solution and incubated overnight in the dark at 25 °C followed by 8 days in the normal growth conditions. Afterwards, the suspension was spread on the cellophane covered BCD solid medium for 3 d and transferred to selection BCD solid medium, pH5.6. The mixture was incubated at room temperature for 30 min and carefully diluted with the gradient volume of 3M medium every 5 min. After centrifuged for 10 min, the transformed protoplasts were resuspended in 3 ml regeneration solution and incubated overnight in the dark at 25 °C followed by 8 days in the normal growth conditions. Afterwards, the suspension was spread on the cellophane covered BCD solid medium for 3 d and transferred to selection BCD solid medium containing antibiotic G418 (25, 50 or 100 mg μL⁻¹) to select for transformants. Moss surviving the third round of selection were regarded as stable transformants, and confirmed by PCR amplification of target fragments using specific gene primer pairs (Table S1).

To construct the transgenic PnSAG1 Arabidopsis plants, the full-length PnSAG1 sequence was introduced into the modified binary pROK2 vector. The constructs were transformed into Arabidopsis wild-type plants by Agrobacterium-mediated floral dip method (Zhang et al., 2006). Transgenic lines and their offspring were selected based on their resistance to kanamycin. Seedlings of T3 or T4 homozygous transgenic lines were selected based on their resistance to kanamycin. Seedlings of T3 or T4 homozygous transgenic lines were subjected to phenotype and molecular analysis. We identified overexpressing lines by extracting genomic DNA and then confirmed the target fragments using PCR amplification.

2.7. Stress assays of transgenic Physcomitrella patens and Arabidopsis plants

For phenotype assays of transgenic P. paten, the same size stem tip of gametophytes of PnSAG1 transgenic and WT plants were cut and grown on BCD agar medium containing different concentrations of NaCl and ABA. After cultured 30 d at 25 °C with 16 h light, the plants size was measured and the visual phenotypes were photographed. The experiments were replicated three times.

For phenotype assays of transgenic Arabidopsis, sterilized seeds were plated on 1/2 MS agar medium supplemented with various concentrations of NaCl and ABA, incubated in darkness at 4 °C for 2 d to break dormancy, and subsequently transferred to a 16 h photoperiod at 22 °C to allow germination. Germination rates were scored as the proportion of seeds that produced open green cotyledons. The seedlings roots length assay was conducted by sowing sterilized seeds on 1/2 MS agar medium containing different concentrations of NaCl and ABA. After growing for 7 d under stress conditions, the root growth was measured. The experiments were replicated three times.

2.8. Statistics analysis

The data of PnSAG1 transcriptional levels, seed germination, early root length and related genes expression were analyzed by a one-way ANOVA followed with Student’s t-test. Error was presented as mean ± Standard Error (SE) and asterisks above columns in the figures indicate statistical differences (*p < 0.05).

3. Results

3.1. Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignments indicated that PnSAG1 (Genbank accession number: MK905235) is homologous with proteins from other species, including Physcomitrella patens (XP_024357944.1), Selaginella moellendorfii (XP_002977628.1), Macleaya cordata (OVA02763.1), Datisca glomerata (AGGS24901.1) and Arabidopsis thaliana (AtPUB14) (Fig. 1b). The amino acid sequence from PnSAG1 shared 84.6% identity with U-box type E3 of P. patens, whereas below 55.0% sequence identity with U-box type E3 of S. moellendorfii, M. cordata, D. glomerata and Arabidopsis. Analyses of the domain architectures revealed that PnSAG1 contained a U-box domain (amino acids 288–351) and four ARM domain (amino acids 418–625), which was first identified in Drosophila Armadillo and functioned in protein-protein interactions (Fig. 1a and b). These findings are consistent with the fact PnSAG1 is an U-box type E3. 64 U-box E3 proteins from higher plant A. thaliana, formed a phylogenetic tree to detect their evolutionary relationship (Fig. 1c). The results showed that PnSAG1 protein was closely related with AtPUB14 of A. thaliana (Fig. 1c).

3.2. PnSAG1 ubiquitin ligase activities and subcellular localization

The subcellular localization of PnSAG1 was investigated in Arabidopsis mesophyll protoplasts. In p35S:GFP-containing protoplasts, GFP fluorescence signal distributed evenly in the cell membrane, cytoplasm, and nucleus (Fig. 2a, v-vii). However, in p35S:PnSAG1:GFP-carrying protoplasts, PnSAG1::GFP signals were exhibited in cytoplasm, which is consistent with the predicted intracellular domain architecture analysis (Fig. 2a, i-iv). These results suggested that PnSAG1 might play a potential role in plant cytoplasm.

To test PnSAG1 ligase activity, we produced PnSAG1 in E. coli as a fusion protein with His-tag and affinity purified PnSAG1-His from the soluble fraction. In the presence of purified AtUBA1, AtUBC8, ubiquitin, ATP and reaction buffer, polyubiquitination activity was detected in the presence of the purified PnSAG1-His protein (Fig. 2b). However, in the absence of E1, E2, ubiquitin, ATP or reaction buffer, no polyubiquitination was observed. These results indicated that PnSAG1 had E3 ligase activity.

3.3. PnSAG1 expression induced by multiple abiotic stresses

To validate whether PnSAG1 is involved in P. nutans response to abiotic stress, RT-qPCR assay was used to analyze the PnSAG1 transcript patterns under multiple environmental stimuli. Under salt stress, the PnSAG1 transcript induction reached a maximum peak with 4.1-fold at 0.5 h and maintained slightly higher induction with 1.7-fold at 3 h (Fig. 2c). Under cold stress, the PnSAG1 expression levels peaked by 1.8-fold at 1 h, declined gradually after that and reached to basal level at 6 h after stress (Fig. 2d). Simulated drought conditions led to abruptly highest transcript accumulation of PnSAG1 at 2 h and remained high up at 6 h after stress (Fig. 2e). Under ABA treatment, PnSAG1 expression was gradually upregulated from 0.5 h to 1 h (Fig. 2f). Increased expression of PnSAG1 by cold, salinity, drought and ABA revealed the stress-responsive character and ABA might mediate regulation of PnSAG1 in P. nutans.

3.4. PnSAG1 reduced salt stress tolerance in transgenic Physcomitrella patens and Arabidopsis

To further illustrate the molecular functions of PnSAG1, we generated the transgenic P. patens and Arabidopsis plants constitutively overexpressing PnSAG1. Three independent transgenic P. patens (#1, #3 and #8) were confirmed by PCR analysis using specific primer pairs (Fig. 3c). To test the effect of PnSAG1 on plant growth during salt stress,
of control and three transgenic lines were treated with varying concentrations of salt (0, 125 and 150 mM NaCl).

Under normal conditions, control and transgenic lines showed equally well growth on BCD medium at 25 °C, and their seedling phenotypes were no obvious differences (Fig. 3a). However, the growth rates significantly decreased in the transgenic gametophytes with clone size of 5.8, 5.4 and 5.4 mm compared with 7.9 mm in control plants at 6 weeks on 125 mM NaCl medium. And the clone size of transgenic gametophytes were 4.4, 4.5 and 3.4 mm compared to 5.9 mm in control plants at 150 mM NaCl medium (Fig. 3b).

The PnSAG1 transgenic Arabidopsis (#3 and #12) were confirmed by RT-qPCR with gene specific primers (Fig. 3h). All three lines showed 100% germination at 22 °C (control). However, following exposure to 125 or 150 mM NaCl, the germination rate of transgenic lines was markedly lower than that of WT plants (Fig. 3d). The germination rates of the transgenic plants decreased 43.4% and 68.7% at 125 mM NaCl, and 51.1% and 64.7% at 150 mM NaCl compared to WT plants, respectively (Fig. 3e). Moreover, in the presence of 125 mM NaCl, the primary root of WT plants was observed to form 2.0-fold longer than the transgenic plants, while there were no phenotypic difference between two transgenic plants and WT plants on standard medium (Fig. 3f and g).

3.5. PnSAG1 enhanced abscisic acid sensitivity in transgenic plants

It has been generally accepted that the plant tolerance to abiotic stresses may connected with ABA signaling pathway. As shown in Fig. 4a, the transgenic P. patens plants (#1, #3 and #8) showed smaller gametophytes size than the control plants after 5 or 10 μM ABA treatment. On 5 μM ABA, the gametophyte sizes of the transgenic P. patens plants were 6.0, 4.1 and 3.5 mm compared to 7.7 mm in control plants, while 3.5, 3.2 and 3.4 mm compared to 5.5 mm in control plants on 10 μM ABA (Fig. 4b).

In Arabidopsis, the germination rate analysis also exhibited that the transgenic plants were insensitive to ABA (Fig. 4c). In the presence of ABA, two transgenic seeds (#3 and #12) germinated slower than WT seeds. The germination rates of transgenic plants were 44.4% and 23.3%, while the germination rate of the WT plants was 70.0% on
0.5 μM ABA. Meanwhile, the germination rates of two transgenic plants were 17.8% and 12.2% compared with 28.9% of the WT plants on 0.75 μM ABA (Fig. 4d). For plants root length analysis, on ABA medium, the primary root growth of two transgenic plants were more impaired than that of WT plants. The root length of the transgenic plants decreased to 27.0% of the WT plants at 0.5 μM ABA (Fig. 4e and f).

3.6. PnSAG1 reduced the expression of stress-responsive genes in transgenic Physcomitrella patens and Arabidopsis

To further determine the possible molecular mechanism of PnSAG1 regulating salinity tolerance, the expression patterns of several stress-responsive genes (PpABI3A, PpABI3B, PpABI3C, PpABI5A, PpABI5B, AtABI3, AtABI4, AtABI5, AtABI3, AtDREB2A, AtNED3, AtRD22 and AtRD29A) was examined by RT-qPCR in the control and transgenic P. patens and Arabidopsis plants. These genes were involved in salt tolerance and ABA signaling pathway in Arabidopsis. After 2 h treatment with 200 mM NaCl, the transcript levels of PpABI3A, PpABI3B, PpABI3C, AtABI3, AtDREB2A, AtABI3 and AtRD29A showed significantly lower transcription accumulations in the transgenic plants than that of the control plants (Fig. 5a and b). However, the transcript levels of PpABI5A, PpABI5B, AtABI3, AtABI5, AtRD22 and AtNED3 have no significant differences compared with WT plants (Fig. 5c and d). These results indicated that the increased salt sensitivity of the
PnSAG1 transgenic lines resulted from the lower expression of these stress responsive genes.

4. Discussion

The U-box type E3 ligases, which could interact with and degrade the specific substrates by ubiquitination, are involved in plant growth and development (Li et al., 2012; Kinoshita et al., 2015; Wang et al., 2017a,b), innate immunity (Liu et al., 2015) and abiotic stress (Trujillo, 2018). In this study, we identified a representative U-box armadillo repeat (PUB-ARM) ubiquitin ligases (PnSAG1) from Pohlia nutans, and it shares relatively high similarity with other PUB proteins including Arabidopsis and the rice (Fig. 1b). Previous studies have shown that the localization of PUB-ARM proteins was diverse, such as in the nucleus and the cytoplasm (BnARC1, TaPUB1, AtPUB30 and AtPUB46), in proteasome structures at the ER (BnARC1), in the cis-Golgi (GmPUB8), and in the plasma membrane (AtPUB30 and AtSAUL1) (Stone et al., 2003; Drechsel et al., 2011; Wang et al., 2016; Zhang et al., 2017a,b; Adler et al., 2018; Peng et al., 2019). Subcellular localization analysis showed that PnSAG1 located in the cytoplasm (Fig. 2a). It is notable

Fig. 4. PnSAG1 increased the ABA sensitivity in transgenic Physcomitrella patens and Arabidopsis. a The size of transgenic Physcomitrella patens gametophyte plants was significantly lower than that of the wild type after ABA treatment (4 week-old plants). b Statistical analysis of gametophyte size as shown in a. c Seed germination of transgenic lines were significantly lower than that of the wild type under different concentrations of ABA (4 days’ seed germination). d Statistical analysis of the transgenic Arabidopsis seedling greening shown in c. e PnSAG1 promotes the growth of Arabidopsis seedling after ABA treatment. f Statistical analysis of the root length in transgenic Arabidopsis shown in e. Vertical bars are means ± SE, and asterisks (*) indicate significant differences of means between the transgenic lines and the WT plants at *p < 0.05.
that functioning as the largest subgroup of E3 ubiquitin ligases (more than 64%), PUB-ARM proteins capable of mediating polyubiquitination (Trujillo, 2018). Polyubiquitination was detected by anti-Ub antibody than 64%), PUB-ARM proteins capable of mediating polyubiquitination that functioning as the largest subgroup of E3 ubiquitin ligases (more than 64%). J. Wang, et al. Plant Physiology and Biochemistry 141 (2019) 343–352

Furthermore, an inducible expression pattern of PnSAG1 was observed under different treatments (ABA, NaCl, PEG and cold stress), which suggests the potential regulatory role of PnSAG1 in plant response to abiotic stress (Fig. 2c).

A previous study revealed that PUB proteins possess biological significance under abiotic stress (Zhang et al., 2015). For example, pub12/pub13 or pub18/pub19 mutants plants were less sensitive to salt- and drought-dependent inhibition of germination in Arabidopsis (Sérgel and Hoth, 2011; Kong et al., 2015). The overexpression of the soybean PUB8 in Arabidopsis hindersensitive to drought and NaCl during seed germination and seedling growth (Wang et al., 2016). Arabidopsis PUB30 negatively regulates salt tolerance probably through facilitating the degradation of BR11 kinase inhibitor 1 (BK11) and brassinosteroids signaling (Zhang et al., 2017c). In the present study, we also found that the PnSAG1-overexpressing Arabidopsis and P. patens both displayed hypersensitivity to salt stress during germination, post-germination growth and root growth (Fig. 3).

The phytohormone ABA has been regarded as a major plants endogenous messenger in controlling cellular and physiological responses to abiotic stresses (Raghavendra et al., 2010). Microarray expression profiles study has suggested that the ABA signaling pathway is conserved among land plants (from bryophytes to higher plants) (Richardt et al., 2010). Multiple ABA signaling transducers are subjected to the regulations by ubiquitination and proteolytic systems (Yu et al., 2016). Several reports have underlined the role of PUBs in ABA signaling process. Plants lacking SAUL1 were impaired in ABA-induced inhibition of germination, suggesting that they are less sensitive to ABA (Salt et al., 2011). In seedlings, PUB18 was the target of Exo70B1 for degradation and, accordingly, exo70B1 plants were less responsive to ABA (Seo et al., 2016). In rice, PUB70 mutants were less sensitive to ABA, whereas AtCHIP may positively regulate PP2A activity (Luo et al., 2006; Samuel et al., 2008). In this study, the transcript levels of stress-inducible genes including AtABI3, AtDREB2A, AtABF3, AtRD29A, PpABI3A, PpABI3B and PpABI3C displayed reduced induction in PnSAG1-overexpressing lines compared with WT plants following salt treatment (Fig. 5a and b). However, the transcript levels of PpABI5A, PpABI5B, AtABI4, AtABI5, AtRD22 and AtNCHED3 have no significant differences compared with WT plants (Fig. 5c and d). These results further support that PnSAG1 negatively regulates salt stress responses. In conclusion, the data presented here provide the phenotypic and genetic evidence in support of a negative role of PnSAG1, a novel U-box domain protein, in ABA and salt stress signaling.

Author contribution statement

PZ, KC and SL conceived and designed research. JW and HL conducted experiments. JW and PZ analyzed data. JW, SL and PZ discussed the results. JW wrote the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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